Reference: *Biol. Bull.*, 159: 219–230. (August, 1980)

STUDIES ON REPRODUCTION IN THE COMPOUND ASCIDIAN, *SYMPLEGMA REPTANS*: RELATIONSHIP BETWEEN NEURAL COMPLEX AND REPRODUCTION[1]

KEIJI SUGIMOTO AND HIROSHI WATANABE[2]

Zoological Institute, Faculty of Science, Tokyo Kyoiku University, Otsuka, Tokyo, Japan

The neural gland in ascidians was first clearly indicated by Hancock (1868). Many morphological or physiological studies have been made on this structure since then. Earlier investigators attributed various functions to the gland: It was postulated to function as a lymphatic organ (Herdman, 1883), an excretory system (Pérès, 1943; Millar, 1953) or a mucus gland (Roule, 1884). In addition, the possibility that it acts as an endocrine organ has been extensively discussed (Butcher, 1930; Carlisle, 1951; Dodd, 1955) since Julin (1881) first postulated a homology of the neural gland of ascidians with the hypophysis of vertebrates on the basis of its position and origin. However, these studies have resulted in much disagreement, and the function and structure of the gland in ascidians remain controversial. Neurosecretory cells have been shown to occur in the neural ganglion of ascidians, one of the main components of the neural complex in this animal (Dawson and Hisaw, 1964; Lane, 1972). Great interest concerns whether the ascidian ganglion has a gonadotropic function (Hisaw *et al.*, 1966; Sengel and Georges, 1966; Bouchard-Madrelle, 1967). However, an acceptable interpretation of function(s) of the neural ganglion and gland has not been proposed.

Many recent studies on the neurosecretory phenomena in various invertebrates other than ascidians have been carried out (Tombes, 1970; Golding 1974, for reviews). Accumulating evidence from such studies indicates that the neurosecretory system in invertebrates plays an important role in reproductive activity. The neurosecretory system may serve a similar function in ascidians. The present study is focused primarily on the gonadotropic function of the neural complex in a compound ascidian, *Symplegma reptans*. As an approach to this subject, the functional criteria for neurosecretory status suggested by Bern (1962) were applied and a possible role for the neural complex in reproductive activity in this species is postulated.

Materials and Methods

Symplegma reptans Oka, a compound ascidian, was used in this study. Colony fragments were attached to glass plates and reared in the bay at the Shimoda Marine Biological Station (presently Shimoda Marine Research Center), Shimoda, Japan.

Zooids with few ova were used for the ganglion-ablation experiment, since gonads of older zooids with several larvae have a tendency to degenerate after isolation from the colony. Operations were done on solitary zooids to avoid possible influ-

[1] Contribution No. 360 from the Shimoda Marine Research Center.

[2] Present addresses: Sugimoto: Department of Anatomy, Nippon Medical School, 1–1 Sendagi, Bunkyo-ku, Tokyo 113, Janpan; Watanabe: Shimoda Marine Research Center, University of Tsukuba, Shimoda 5–10–1, Shizuoka-ken 415, Japan.

ences from unoperated zooids in a colony. Zooids were made solitary on the glass plates by removing other zooids and, thereafter, newly formed buds. A small incision was made along the neural complex through the tunic and the ganglion was cut off with a tungsten wire with its tip bent in a hook. Control zooids received only the incision. To confirm completeness of ganglion removal, physiological response of the operated zooids to exogenous stimuli was examined by touching a needle to the buccal aperture, which does not contract quickly in a zooid having no ganglion. Histological examinations were also made. The operated zooids were hung upside-down so that their excretions could easily pass through an atrial siphon.

Electrical stimulation of the ganglion was carried out to release neurosecretory materials from neurosecretory axon-terminals into the circulatory system. After surgical exposure of the ganglion as described above, electrical shocks were administered to the ganglion, which was sucked into a glass needle containing the stimulatory electrode (platinum wire, 100 μ in diameter). Rectangular pulses of 10–150 V amplitude and 1–5 msec duration were applied at the rate of 2–50 shocks per sec for 15–30 min. Controls received only the operation without the electrical shocks. Effects of the stimulation on the neurosecretory cells in the ganglion were examined histologically by applying Gomori's paraldehyde-fuchsin (Gomori's stain) to the fixed materials within 1 hr after stimulation. Small colonies composed of six to seven sexually mature zooids and one to two sexually immature zooids were used in bioassays to examine the physiological effect of the stimulation on the growth of oocytes. Electrical stimulation was done on mature zooids and the growth rate of oocytes in immature zooids was recorded.

For histological study, materials were fixed in 2.5% phosphate-buffered glutaraldehyde at pH 7.4 for 2.5 hr, followed by 1% phosphate-buffered osmium tetroxide at pH 7.4 for 1 hr. Materials then were dehydrated in ethanol series and embedded in Epon. To calculate numbers of neurosecretory cells in the ganglion, sections were prepared every 3 μ on a Porter Blum ultramicrotome and stained with Gomori's stain after removal of resin according to the method of Imai *et al.* (1968). Thin sections were stained with uranyl acetate and lead citrate and examined in a Hitachi HS-9 electron microscope at 50 kV.

Results

Structure of the neural complex in Symplegma

The components of the neural complex in *Symplegma* are the same as those in other ascidians: ganglion, neural gland, and hypophysial duct. The cells of the neural gland have a long axis of about 12 μ and range from cuboidal to irregular in contour. The glandular cells often appear to be vacuolated and were not specifically stained by Gomori's stain. Electron micrographs of the cells were characterized by a number of large cytoplasmic vacuoles filled with fibrous materials. Structural connections between the neural gland and the ganglion were not observed. The cells of the hypophysial duct did not stain at all with Gomori's stain.

The ganglionic cells are relatively small, measuring about 6–7 μ in long axis, and surround a central core of nerve fibers. Some of them were positively stained by Gomori's stain, as has been shown in other ascidians. Furthermore, they could be classified ultrastructurally into three types by cytoplasmic contents: 1) Cells having abundant mitochondria and no granular components. 2) Cells having numerous electron-dense granules 100–140 mμ in diameter (Fig. 1-A). 3) Cells having electron-transparent granules 240–300 mμ in diameter (Fig. 1-B). The

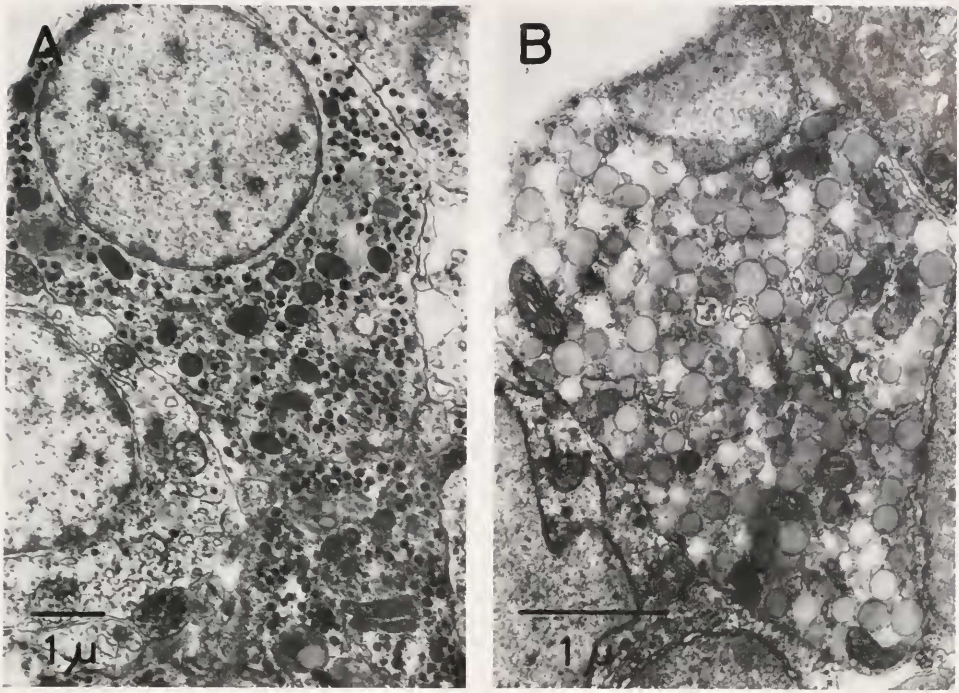

FIGURE 1. Two types of neurosecretory cells in the ganglion: (A) neurosecretory cells containing electron-dense small granules with diameters of 100–140 mμ. (B) a neurosecretory cell containing electron-transparent granules with diameters of 240–300 mμ.

latter two cell types have features characteristic of neurosecretory cells described in many other animals. The last cell type could not be distinguished histochemically from the second cell type and was rarely observed in electron micrographs. Almost all neurosecretory cells in the ganglion belong to the second cell type.

Axon bundles containing the same types of granules as in nuerosecretory cells were observed often only near the ganglion. The bundles were located just beneath the epidermis (Fig. 2). Other bundles of unmyelinated nerve fibers ran deeper and contained no granular components. The axon terminal of each neurosecretory cell or neurohemal organ, where a number of neurosecretory cells' axons terminated, was not found in the present study.

Relationship between the neurosecretory cell and gonad development

During early gonad development, ganglionic cells showed little cytoplasmic specialization for producing granules. Only a few unidentified granules were found near the poorly developed Golgi apparatus. Gomori-postive-staining cells were not found in the ganglion at this stage. When oocytes attained 50 μ in diameter, still in the stage of previtellogenesis, some ganglionic cells produced cytoplasmic granules and reacted positively with Gomori's stain. Such cells increased in number with oocyte development. Their rapid increase was particularly noticeable in the period when the eldest oocyte grew from the stage of previtellogenesis to vitellogenesis. After this stage, the number of cells remained constant (Fig. 3).

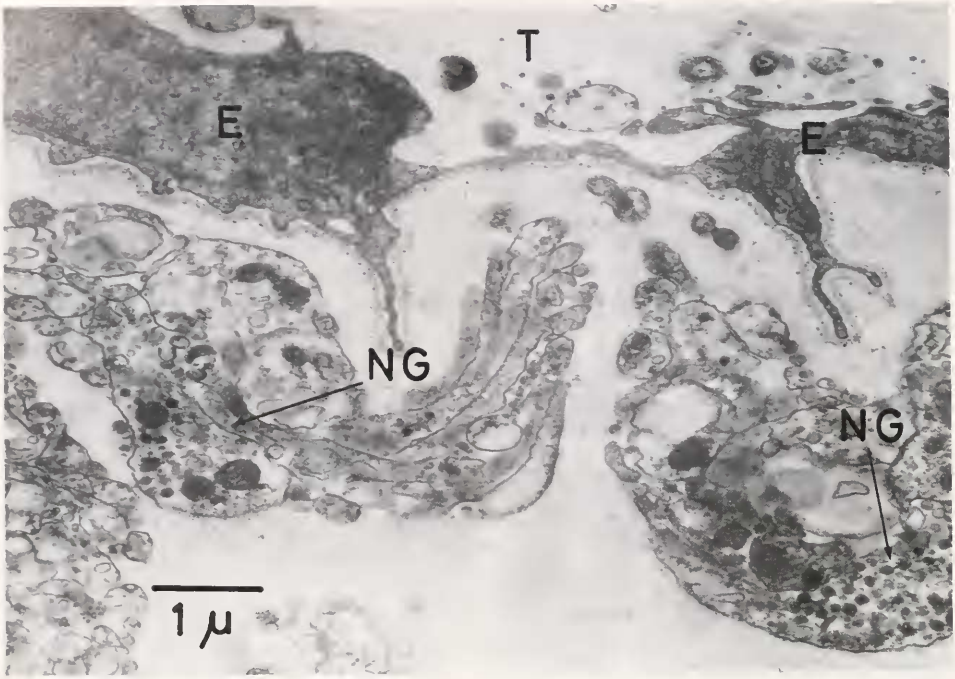

FIGURE 2. Axons distributed near the ganglion. Note the axons containing neurosecretory granules. E = epidermis; NG = neurosecretory granule; T = tunic.

The relationship between the neurosecretory cells and gonad development was also investigated under conditions disadvantageous for growth of oocytes. First, the number of Gomori-positive cells was examined in young solitary zooids. Zooids having small oocytes, 30 μ in diameter, were isolated and stripped of buds. During the 15 days after isolation, their oocytes grew little and remained previtellogenic, 30–50 μ in diameter. Oocytes of same-aged zooids in the colony that served as control attained 100–150 μ in diameter during the same period. The number of Gomori-positive cells in the experimental zooids averaged 35.5 ± 8.9 cells per ganglion. That of control zooids was 112.0 ± 13.9 cells per ganglion (Fig. 4), the same as in isolated, sexually mature zooids with several larvae in their brood sacs. Although the experimental zooids continued growth and bud formation, their gonads degenerated completely by 7 days after isolation. The number of Gomori-positive cells decreased linearly and reached about half that of the control zooids in 10 days (Fig. 4). With electron microscopy, many lysosomes were observed in the neurosecretory cells of ganglia at this stage, and a number of specific granules were contained in such structures (Fig. 5).

Effects of ganglion ablation on gonad development

The growth rate of zooids decreased slightly by 3–5 days after ganglion ablation, but thereafter increased in both control and experimental groups (Fig. 6). Here, small oocytes less than 70 μ in diameter and in the stage of previtellogenesis are called Stage 1, and large vitellogenic oocytes 70–200 μ in diameter are called Stage 2. Stage 1 oocytes decreased to as few as 75% of the initial number, but their complete disintegration was not observed (Fig. 7-A). On the other hand,

Stage 2 oocytes were greatly damaged by the operation and their number decreased to less than half within one day and approached zero by 10 days after operation (Fig. 7-B). Neither fully grown ova, which were more than 200 μ in diameter, nor testes were affected by ganglion ablation. The ova in operated zooids ovulated normally into the brood sacs.

Effects of electrical stimulation of the ganglion on gonad development

Attempts to transplant ganglia into a ganglion-ablated zooid were unsuccessful. In place of transplanting experiments, electrical stimulation of the ganglion was carried out. Rectangular pulses of 40 V amplitude and 5 msec duration were applied at 10–20 shocks per sec for 15 min. Under these conditions, the Gomori-positive cells in the ganglion decreased in number to about 50% of control. The number of cells in the stimulated zooids averaged 56.7 ± 23.1 cells per ganglion and that of control zooids averaged 110.8 ± 15.0. The possibility that electrical stimulation to the ganglion accelerated release of granules from the neurosecretory cells led us to examine the effects of ganglion stimulation on gonad development. Six to seven sexually mature zooids were stimulated electrically as described above. If oocytes of the immature zooids were still in the stage of previtellogenesis, growth of such oocytes was accelerated by the stimulation (Fig. 8-A). On the eighth day after stimulation, oocytes in the unstimulated controls averaged 111.4 μ in diameter, while oocytes in the stimulated group averaged 142.3 μ in diameter. This difference was statistically significant. On the other hand, if oocytes of the

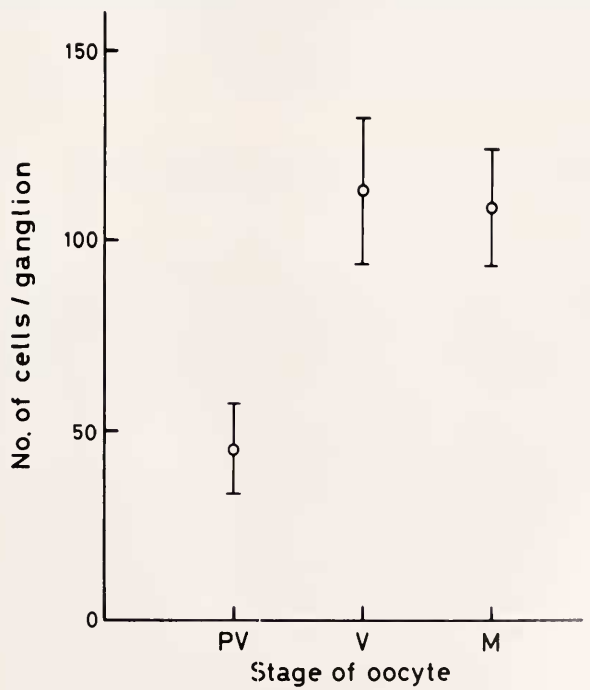

FIGURE 3. Variation in the number of Gomori-positive-cells in the ganglion at different stages of gonad development. Means and ranges of estimated cell numbers per ganglion in 13 zooids at each developmental stage are depicted. PV = previtellogenic; V = vitellogenic; M = fully grown ova.

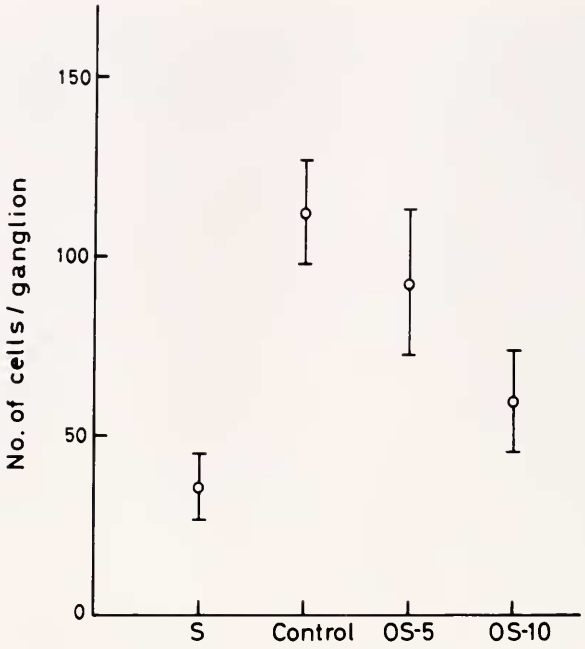

FIGURE 4. Variation in number of Gomori-positive cells under conditions disadvantageous for gonad development. Ten young zooids with previtellogenic oocytes and 10 old zooids with several larvae were isolated from the colony and reared alone for 15 and 10 days, respectively. Thirteen zooids with vitellogenic oocytes served as controls. Means and ranges of estimated cell numbers per ganglion in each zooid are depicted. S = young solitary zooid; OS-5 = 5-day-old solitary zooid after isolation; OS-10 = 10-day-old solitary zooid after isolation.

immature zooids were vitellogenic, growth of these oocytes was almost the same in both the stimulated and control groups (Fig. 8-B).

DISCUSSION

Although many morphological and physiological studies on the neural gland of ascidians have been made, the functions of this structure have not yet been fully resolved. No intimate structural relationship between neural gland and neural ganglion has been observed in *Symplegma*. Moreover, cells with membrane-bound secretory granules, which characterize the secretory cells of vertebrate adenohypophysis, have not been found in the neural gland. Instead of such granules, a number of vacuoles are found in the glandular cells, as reported by Lane (1971). The fact that degenerating cells show a high degree of cytoplasmic vacuolation suggests that the glandular cells may secrete their products by holocrine secretion, as do sebaceous or meibomian glands in vertebrates. Histological characteristics reveal that the glandular cells are distinctly different from typical neurosecretory cells in many species. In connection with secretory cells, it is interesting that neurosecretory cells occur in the neural ganglia of ascidians (Dawson and Hisaw, 1964; Chambost, 1966; Lane, 1972), which lies immediately adjacent to the neural gland. In *Symplegma*, two types of neurosecretory cells can be distinguished by electron microscopy. The character of granules surely is a reflection of function, but whether these cells are concerned with different functions or not must be discerned in the future.

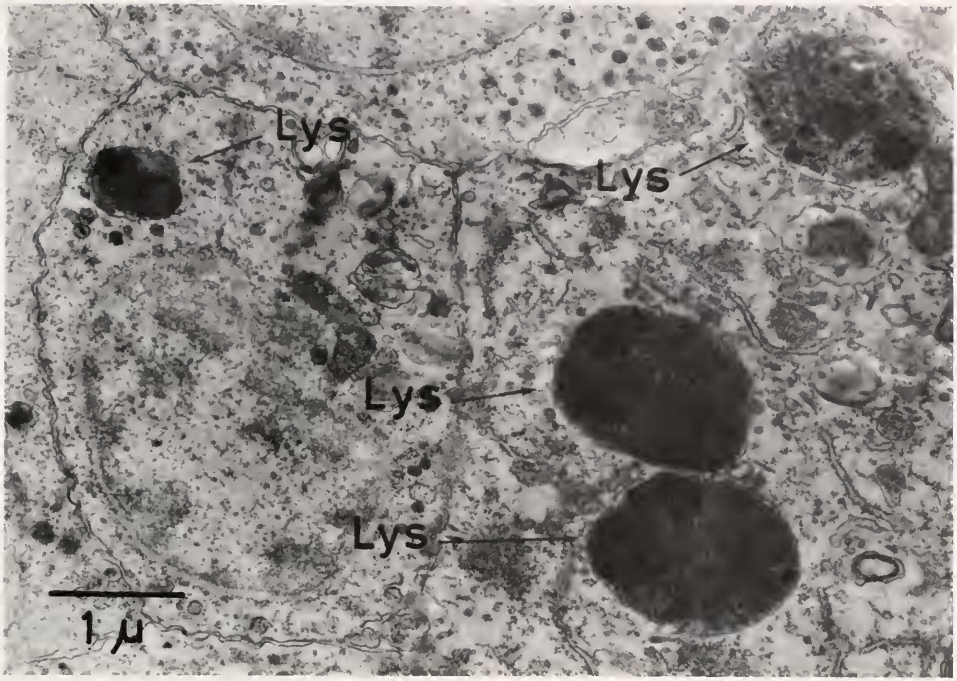

FIGURE 5. Secondary lysosomes appearing in the neurosecretory cells of 10-day-old solitary zooids after isolation. Lys = secondary lysosome.

A neurohemal region where neurosecretory materials are released into the circulatory system could not be identified in the present study. Axonal endings of neurosecretory cells may exist near the ganglion, since axons containing the neurosecretory granules were found only near the ganglion. A well-defined neurohemal organ such as the median eminence of vertebrates, the sinus gland of crustaceans, and the corpus cardiacum of insects was not found in *Symplegma*. Such diffuse and short-range distribution of the axon terminals is known in the ganglia of molluscs (Frazier *et al.*, 1967; Toevs and Brackenburry, 1969; Loh *et al.*, 1973).

Many studies concerning the mechanism of reproduction in ascidians have been carried out since Julin (1881) postulated a homology of the neural gland with the hypophysis of vertebrates. Previous studies examining possible gonadotropic potency of the neural complex showed contradictory results: Butcher, 1930; Hogg, 1937; and Carlisle, 1951, concluded that there was a positive function but Dodd, 1955, and Sawyer, 1959, showed negative function. However, the bioassay system of earlier investigators, using tissues of vertebrates, has to be reconsidered because there is no reason to suppose that similar phenomena in such phylogenetically distinct organisms are regulated by the same materials. For example, gonadotropin and progesterone affect oocyte maturation in vertebrates, but the same phenomenon in starfish is regulated by a peptide hormone from the nervous system and 1-methyladenine (see Kanatani, 1973, for review). Therefore, in examining hormonal activity of a species, animals of at the very least the same phylum must be used in order for bioassays to have relevance.

Many studies concerning the function of neurosecretory cells on gonad development indicate that distinctly contrasting control mechanisms operate in various

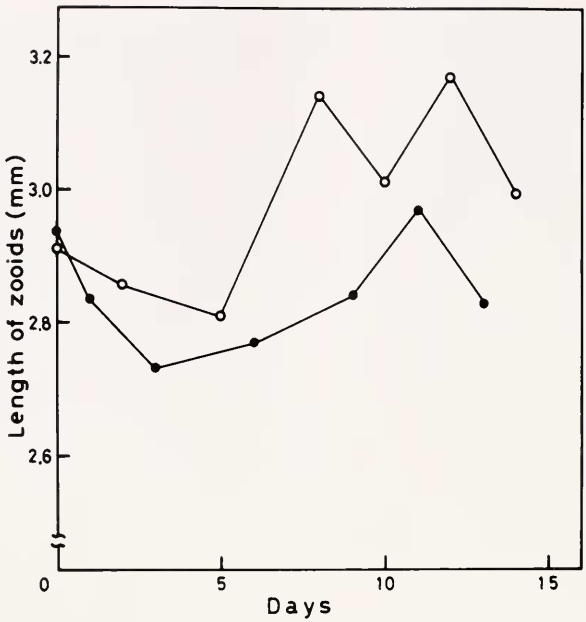

FIGURE 6. Effects of ganglion ablation on growth of zooids. Average growth curves of 35 operated zooids (solid circles) and 27 control zooids (open circles) are depicted.

species. For example, control of sexual reproduction by the neurosecretory system in some species is inhibitory in character, whereas a positive gonadotropic influence exists in other species. Neurosecretory materials in *Hydra* act somewhat like a growth hormone and inhibit gonad development (Schaller, 1973; Schaller and Gierer, 1973). Clearly, neurosecretory cells in *Hydra* affect asexual reproduc-

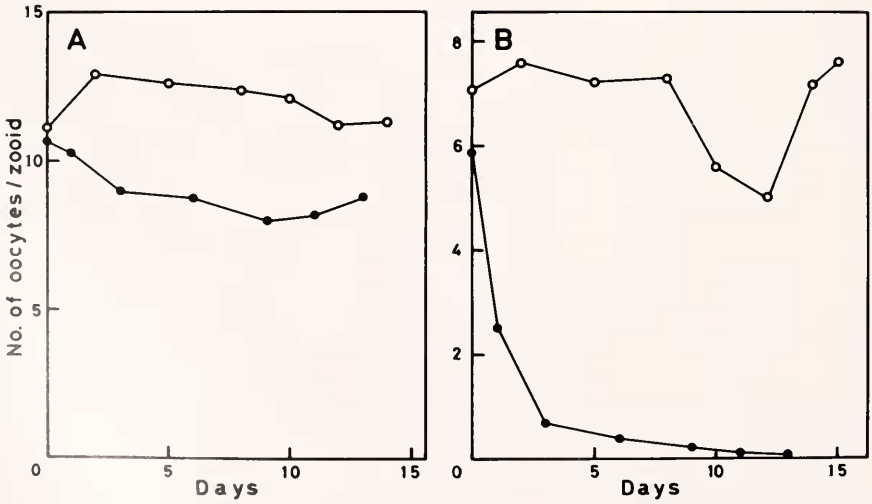

FIGURE 7. Effects of ganglion ablation on oocyte growth. Average oocyte number in 35 operated zooids (solid circles) and 27 control zooids (open circles) are depicted: (A) variation in the number of oocytes in the stage of previtellogenesis; (B) variation in the number of vitellogenic oocytes.

tion, that is, zooid growth and bud formation. The same inhibitory influences of neurosecretory cells were shown in nemertines (Bierne, 1970) and in nereid polychaetes (Clark, 1965; Baskin and Golding, 1970). On the other hand, a positive function has been demonstrated in many other invertebrates, such as turbellarians (Grasso and Quaglia, 1971), molluscs (Geraerts and Algera, 1976; Wijdenes and Runham, 1976), non-nereid polychaetes (Howie, 1966; Gouedard-Couadou and Vicente, 1971), and arthropods (Tombes, 1970; Adiyodi and Adiyodi, 1970 for reviews). In *Symplegma,* the pattern of occurrence of Gomori-positive neurosecretory cells is different from that of *Hydra.* Active zooid growth and bud formation were observed in both young and aged zooids isolated from the central region of a colony, but they showed only a few such neurosecretory cells in their ganglia. As shown in Figure 3, variation in the number of Gomori-positive neurosecretory cells is superficially related to oocyte development. That is, the cell number increases rapidly when the oocyte proceeds from previtellogenesis to vitellogenesis.

In addition, these cells decrease in number under conditions disadvantageous for gonad development. Thus, the neurosecretory cells in *Symplegma* seem to be functionally insignificant in asexual reproduction but to have some significance in sexual reproduction. If this is so, surgical removal of the ganglion should affect gonad development. The most distinctive effect of the operation was the breakdown of oocytes in Stage 2, while young Stage 1 oocytes were mildly affected by the operation. Similar results have been obtained in *Ciona* (Bouchard-Madrelle, 1967). But studies in *Chelyosoma* showed that neural-complex ablation has no effect on gonad development (Hisaw *et al.*, 1966). Additional studies to compare single ascidians with compound ones or to compare species with distinct

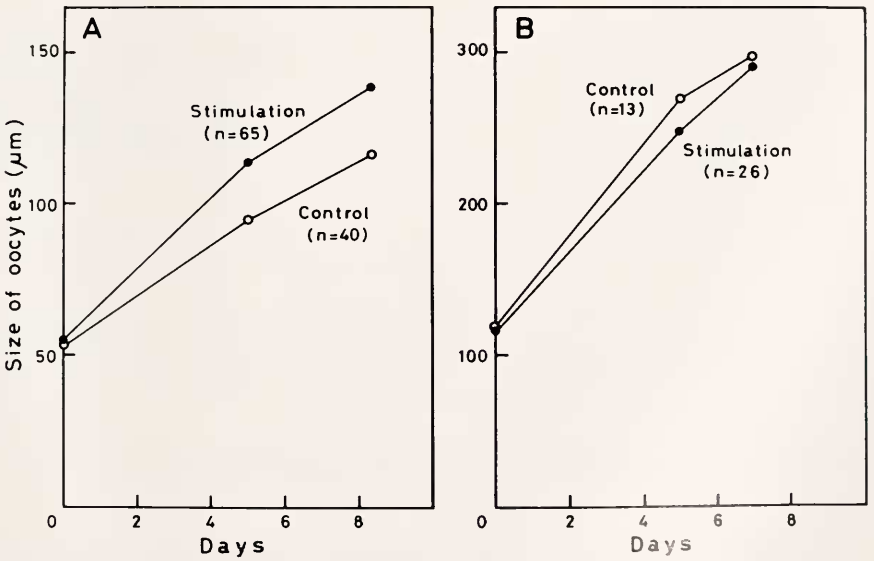

FIGURE 8. Effects of electrical stimulation of the ganglion on the growth of oocytes. Average growth curves of oocytes in the stimulated and control groups are depicted. Figures in parentheses show the number of oocytes used in each group: (A) growth of oocytes in the stage of previtellogenesis; (B) growth of vitellogenic oocytes.

breeding seasons with those without limited breeding seasons will be needed to elucidate the differences between them.

As shown in Sugimoto and Watanabe (1980) delay of oocyte development in zooids reared from their early developmental stages in isolation may result not from the slow rate of synthesis of vitelline, but rather from prolongation of the previtellogenic period. This finding and the results in the present study suggest that initiation of vitellogenesis may be the critical point in oocyte maturation. If we accept this view, the effect of electrical stimulation on the growth of young oocytes in the stage of previtellogenesis can be interpreted as indicating that the stimulation speeds the initiation of vitellogenesis. Similar effects of electrical stimulation on gonad development have been shown in insects (Highnam, 1962, Wigglesworth, 1964).

Thus, the ganglion in this species may have an important role in the process of vitellogenesis. To substantiate this possibility, it is necessary to examine the dynamics of neurosecretory materials at each stage of gonad development, using an *in vitro* system to see whether or not neurosecretory materials act directly on the gonad.

We are grateful to Mr. Masahiko Sato, Dartmouth College, for his helpful suggestions and a critical reading of the manuscript. We wish to acknowledge our indebtedness to the staff of the Shimoda Marine Research Center for their kindness in providing many conveniences throughout the course of the study. This study was supported, in part, by a grant-in-aid for Scientific Research from the Ministry of Education of Japan.

Summary

1. The neural complex was examined for induction of sexuality in a compound ascidian, *Symplegma reptans*. The number of Gomori-positive neurosecretory cells in the neural ganglion increases rapidly while oocyte development proceeds from previtellogenesis to vitellogenesis and is constant in sexually mature zooids. However, such cells decrease in number in conditions disadvantageous for gonad development. The decrease in cell numbers in isolated aged zooids may result from lysosomal digestion of granules.

2. Fully grown ova and previtellogenic oocytes suffer mild influences from ganglion ablation. However, vitellogenic oocytes always suffer profound damage from the operation.

3. In sexually mature zooids, electrical stimulation to the neural ganglion leads to decrease in the number of Gomori-positive neurosecretory cells. The stimulation accelerates growth of previtellogenic oocytes, but does not affect oocytes in other developmental stages.

4. These findings suggest that the ganglionic neuroscretory cells in *Symplegma* may function in sexual reproduction, especially in the vitellogenesis.

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